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Quantitative RT-PCR

Paul D. Siebert

1. Introduction and Overview

Although reverse transcriptase polymerase chain reaction (RT-PCR) is an extremely sensitive method of mRNA analysis, obtaining quantitative information with this technique can be difficult. This is caused primarily by the fact that there are two sequential enzymatic steps involved: the synthesis of DNA from the RNA template and PCR. In practice, the exponential nature of PCR and the practical aspects of performing PCR pose the most serious obstacles to obtaining quantitative information. With some adaptations, however, RT-PCR can yield accurate quantitative results.

This chapter describes a number of methods that have been developed for using RT-PCR to determine the relative level of abundance of a particular mRNA, changes in the abundance of an mRNA over time or after induction, and the actual number of mRNA molecules in the sample. The theory and applications of each method are discussed, as well as the advantages and limitations associated with them. The chapter then expands on one method of quantitative PCR in particular, namely competitive PCR. This method, that uses nonhomologous internal standards (PCR MIMICs), is both simple and useful.

2. Theoretical and Practical Aspects of PCR

2.1. The Exponential Nature of PCR

By definition, the PCR process is a chain reaction. The twofold increase in products from one cycle of amplification serve as substrates for the next. Therefore, the amount of product increases exponentially and not linearly, as in most enzymatic processes. Under ideal or theoretical conditions, the amount of product doubles during each cycle of the PCR reaction according to Eq. 1. This relationship is plotted in Fig. 1A.

$$N = N_0 2^n \quad (1)$$

From: *Methods in Molecular Medicine, Vol 26: Quantitative PCR Protocols*
Edited by: B. Kochanowski and U. Reischl © Humana Press Inc., Totowa, NJ

999 Riverview Drive, Suite 208
Totowa, New Jersey 07512

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Cover illustration: Fig. 1 from Chapter 16, "High Resolution PCR Quantitation by AmpliSensor Assay," by Chang-Ning Wang.

Cover design by Patricia F. Cleary.

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Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1

Library of Congress Cataloging in Publication Data

Main entry under title:

Methods in molecular medicine™.

Quantitative PCR Protocols / edited by Bernd Kochanowski and Udo Reischl.
p. cm. -- (Methods in molecular medicine™ ; 26)
Includes index.
ISBN 0-89603-518-2 (alk. paper)
1. Polymerase chain reaction--Laboratory manuals. I. Kochanowski, Bernd. II. Reischl, Udo.
III. Series.
[DNLM: 1. Polymerase Chain Reaction--methods. 2. DNA--analysis. 3. RNA--analysis.
QH 450.3 Q11 1999]
QP606.D46Q36 1999
572.8--dc21
DNLM/DLC 99-10332
for Library of Congress CIP

Preface

Since the polymerase chain reaction (PCR) was first developed in 1985, an enormous number of research reports have documented the versatility of this brilliant technique for in vitro amplification of nucleic acids. Although PCR has had a profound impact in many areas of research, contrary to expectation its routine application to the quantitation of nucleic acids has proven problematic in several aspects. The shortcomings are principally caused by the exponential nature of PCR, whereby small variations in amplification efficiency may dramatically affect the yield of amplification product. Even minimal temperature deviations that occur between adjacent wells of a thermocycler or day-to-day variations in the efficiency of nucleic acid preparation can lead to significant differences in the extent of amplification between otherwise identical samples.

However, knowing more about the intrinsic limitations of PCR is the first step towards surmounting the shortcomings associated with this promising methodology. With the introduction of appropriate standards of known amount, which are co-amplified with the sample using the same primers, it is increasingly feasible to address biological or diagnostic questions that are difficult or impossible to answer using any other experimental approach.

The techniques and experimental strategies described in *Quantitative PCR Protocols* are representative of those most generally applicable to routine work at present. Apart from a brief description of the principles of quantitative PCR, the book describes both established and novel strategies, each of which has been applied successfully to such problems as the analysis of eukaryotic gene expression, the quantitation of viral loads in clinical specimens, reporter gene expression, and quantitative oncogene analysis.

Particular emphasis is placed on the underlying principles of the design of competitive or noncompetitive standards, as well as the optimization of the amplification process; these are crucial in any successful quantitative application. Basic problems with the interpretation of the results are addressed as well. Some duplication of important topics has been introduced purposely to offer the reader several approaches to the same problem. It is hoped that this collection of detailed protocols, providing comprehensive and up-to-date information, will be especially useful to researchers and to students needing

to become familiar with the principles of quantitative PCR, and guiding them to set up test systems tailored to their specific practical needs. Since approaches to the amplification of nucleic acids in a quantitative manner and to the technology involved in product detection are subject to continual improvement, *Quantitative PCR Protocols* does not attempt the impossible task of treating every variety of experimental approach in the field. Rather, it depicts a kind of cross-section of realistic possibilities for the user's conception of still more refined assays.

Quantitative PCR—myth or reality? At the present moment the truth lies somewhere in-between and, since the usefulness of quantitation mainly depends on the particular application, only the future will show which assays will prove most useful in individual diagnostic situations.

We are especially indebted to Prof. Hans Wolf and Prof. Wolfgang Jilg for giving us the opportunity to gain substantial experience in the field. Without their confidence and continuous support many things would not have been possible. We also thank Prof. John Walker for his encouragement and Humana Press for their excellent assistance during the assembly of this volume. Finally, we are grateful to all of the contributing authors for their constantly high level of motivation and enthusiasm and, last but not least, for providing such good manuscripts.

Bernd Kochanowski
Udo Reischl

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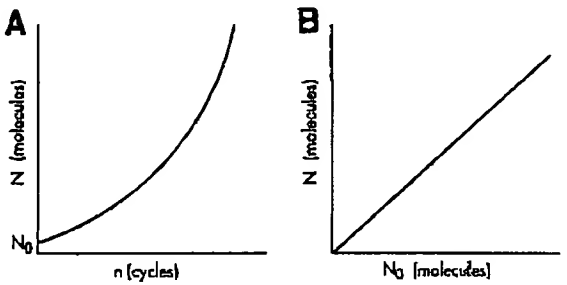

Fig. 1. Characteristics of PCR amplification in an ideal case. (A) Kinetics of amplification and (B) PCR product yield as a function of initial amount of target.

where N is the number of amplified molecules, N_0 is the initial number of molecules, and n is the number of amplification cycles. **Eq. 1** indicates a linear relationship between the number of amplified target molecules and the initial number of target molecules. This relationship is shown in Fig. 1B.

2.2. The Efficiency of Amplification

Amplification efficiency, that is, the fraction of the template replicated during each reaction cycle, is a crucial factor for any reliable method of quantitative PCR. Experimentally, the efficiency of amplification (E) is less than one, and the PCR process is thus described by **Eq. 2**.

$$N = N_0 (1 + E)n \quad (2)$$

where E is the amplification efficiency. Because of the exponential nature of PCR, a very small change in amplification efficiency, E, can yield dramatic differences in the amount of product, N, even if the initial number of target molecules, N_0, is the same. For example: if $E = 0.85$ and $n = 30$, then $N = N_0 (1 + 0.85)^{30}$ and $N = 10.4 \times 10^7 N_0$. In other words, with 85% efficiency, 30 cycles of PCR would produce a 10.4×10^7-fold increase in the amount of target molecules. However, if E is reduced to 0.8, the target would only be amplified 4.6×10^7 times by PCR. Thus, a change in amplification efficiency of only 0.05 would produce a greater than twofold change in the amount of reaction product. This difference becomes even greater as the number of cycles increases.

Several experimental factors may affect the efficiency of amplification, including:

1. The sequence being amplified.
2. The sequence of the primers.
3. The length of the sequence being amplified.
4. Impurities in the sample.

The first three of these factors are important because they affect secondary structure formation and the G/C content of the target sequence—both of which may interfere with primer binding, affect the melting point of the target sequence, and reduce the processivity of the polymerase. The length of the target sequence being amplified can affect E for another reason: Even with an ideal template, no polymerase exhibits 100% processivity under in vitro conditions. Because of the limited processivity of *Taq* DNA polymerase in vitro, target sequences longer than 3 kb are extremely difficult to amplify. More importantly, there is also some controversy about whether differences in target sequence lengths significantly alter the efficiency of amplification when the sequences are <1 kb. In two cases, a weak inverse correlation was observed *(1,2)*. In another case, there was no observed difference in E *(3)*. Impurities in the sample can affect amplification efficiency in many ways. For example, they can degrade or inhibit the polymerase, cause conformational changes in the target DNA, or compete for primer binding sites—to name just a few of the possibilities.

There may be additional, unknown, subtle factors that affect E. This is illustrated by the fact that the amount of product amplified from the same target sequence after the same number of cycles and under identical experimental conditions often differs from one PCR reaction to another. This was seen even when using a master mix of reaction components *(4,5)*. Unfortunately, such tube-to-tube variation in amplification efficiency can be both significant and unpredictable. Theoretically, the efficiency of amplification, E, ranges from 0–1. Experimentally, the value of E has been found to range from 0.46–0.99 for different genes *(3,6)*. The value of E also varied, from 0.8–0.99, when the same gene was amplified in independent tubes under identical conditions *(7)*.

2.3. The Plateau Effect

Experimentally, the amount of product generated during PCR also deviates from the theoretical case. The amount of PCR products produced during the PCR initially increases exponentially, but then the rate of production slows and finally levels off, as shown in **Fig. 2A,B**. **Fig. 2A** is a graph of the number of amplified target molecules (N) plotted as a function of PCR cycles (n), and Fig. 2B is a graph of the number of amplified target molecules (N) plotted as a function of the initial number of target molecules (N_0). The leveling off of the rate of amplification is often referred to as the plateau effect.

The following factors can contribute for the observed plateau effect:

1. The product accumulates to a concentration at which reassociation competes with primer annealing and extension *(8)*.
2. The molar ratio of polymerase to template falls below a critical value.
3. Inhibitors of polymerase activity, such as pyrophosphates, may accumulate.
4. One or more of the components necessary for the reaction become limiting.

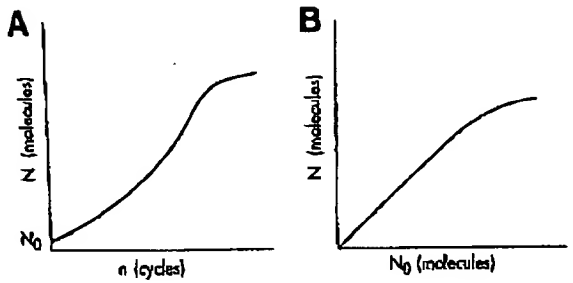

Fig. 2. Characteristics of PCR amplification in a typical case. (A) Kinetics of amplification and (B) PCR product yield as a function of initial amount of target.

The number of cycles needed to reach the plateau phase varies, depending on the sequence—and the original amount—of target mRNA. This variability makes it difficult to predict precisely the time-course of the reaction or the amount of product synthesized before plateau phase is reached. The uncertainties inherent in the plateau effect, as well as the exponential nature of PCR, contribute to the difficulty of performing quantitative PCR because they obscure the linear relationship between N_0 and N depicted in **Eqs. 1** and **2**. Methods that employ RT-PCR to obtain quantitative information must take these factors into account.

2.4. Quantifying the PCR Product

The goal of quantitative PCR is to deduce, from the final amount of PCR product, either the initial number of target molecules (N_0) or the relative starting levels of target molecules among several samples. Thus, the first step in this process is to measure the amount of PCR product present.

Several methods are commonly used to quantify PCR products. The most straightforward approach is to measure the incorporation of labeled nucleotides or primers into PCR products resolved by gel electrophoresis. Although direct, the use of labeled nucleotides in PCR can be problematic. High levels of unincorporated, labeled nucleotides in the PCR product mixtures result from the relatively high (to 100 μM) concentrations of nucleotides required for PCR. Consequently, trace amounts of unincorporated label often remain in the electrophoretic gel as the product bands migrate, resulting in a "trail" of label throughout the lane. Even a relatively small amount of "trailing" can make it difficult to measure the amount of incorporated label. For this reason, many researchers prefer to use labeled PCR primers rather than labeled nucleotides.

Other strategies for quantifying PCR products are based on hybridization. The most common of these methods is to probe a Southern blot of the PCR products using a radioactively labeled probe complementary to the specific, amplified

sequences. To quantitate the amount of probe hybridized, the blot can either be exposed to X-ray film and the resulting autoradiogram densitometrically scanned, or the PCR product band can be excised from the blot and its radioactivity measured in a scintillation counter. Because the nucleic acid probes only hybridize to the corresponding amplified DNA sequences, this method offers the advantage of detecting only the correct PCR product. Nonspecific products do not produce a signal.

Alternative hybridization methods that avoid Southern blotting have also been utilized *(5,9,10)*. Jalava et al. *(9)* described an approach based on the capture and hybridization of biotinylated PCR products on streptavidin-coated microtiter plates. The biotin group is added to the PCR product during amplification through the use of a biotinylated primer. Biotinylated products are subsequently captured on streptavidin-coated plates, and a radioactively labeled nucleic acid probe, complementary to the biotinylated strand, is then used to measure the amount of captured product. Jalava et al. used relatively long, nick-translated DNA fragments (0.35 and 0.42 kb) as the radioactive hybridization probes; however, the results of their experiments suggest that it might also be possible to use short, nonisotopically labeled synthetic DNA probes in conjunction with an appropriate detection system.

Another hybridization method that avoids Southern blotting is solution hybridization of a radioactively labeled probe and denatured PCR products. The hybridized probes are resolved by gel electrophoresis and subsequently quantitated by scintillation counting *(5)*. Fluorescent labels also can be used instead of radioactivity. In this case, a fluorescently labeled internal primer is annealed to one strand of the PCR product and extended using *Taq* DNA polymerase. Run-off extension products are electrophoresed in an automated DNA sequencer that quantitatively detects the incorporated fluorescent label *(10)*.

Several additional methods exist for quantifying PCR products. They include measurement of the EtBr luminescence emanating from PCR products resolved by gel electrophoresis *(11)*, use of high-performance liquid chromatography *(12)*, and assays based on in vitro transcription with radioactively labeled ribonucleotide substrates *(13)*. For in vitro transcription, a transcriptional promoter is incorporated into one of the PCR primers. Following amplification, the PCR product is transcribed in vitro using radioactively labeled ribonucleotides. During transcription, the radioactive signal is amplified 100- to 200-fold, making this a very sensitive detection method. However, the additional enzymatic reaction required for in vitro transcription makes this one of the more laborious detection methods and may also increase the risk of experimental error.

3. Quantitative PCR Without the Use of Internal Standards

Most commonly, researchers use internal standards to control variations in amplification efficiency and to determine absolute values of mRNA (discussed

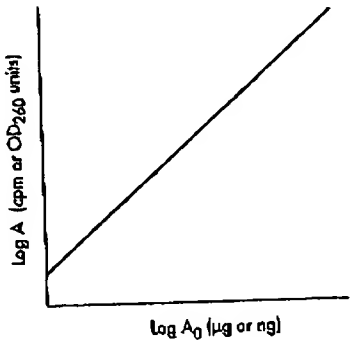

Fig. 3. Linear relationship between the log of the amount of PCR product and the log of the initial amount of sample RNA (µg) or cDNA (ng), in an ideal case.

in **Subheading 3.1.**). However, it is possible to perform quantitative PCR without internal standards if two conditions are met. First, tube-to-tube variation in the actual value of E must be minimal so that a constant value can be assumed for E in all related PCR reactions. Second, all data must be obtained before the reactions begin to reach the plateau phase. The methods described in this section employ mathematical models based on **Eqs. 2** and **3** to determine relative changes in mRNA levels.

$$\log N = [\log (1 + E)]\, n + \log N_0 \quad (3)$$

where N is the number of amplified molecules, N_0 is the initial number of molecules, n is the number of amplification cycles, E is the amplification efficiency.

For convenience, Eqs. (2) and (3) may also be written as:

$$A = A_0 (1 + E)n \quad (2.1)$$

$$\log A = [\log (1 + E)]\, n + \log A_0 \quad (3.1)$$

where A is the amount of amplified product (in cpm or OD_{260} U), and A_0 is the starting amount of total RNA (µg) or cDNA (ng). Note: the target sequences usually comprise only a small fraction of the total. At the end of this section is a discussion of the use of linear regression analysis (also based on these equations) to estimate absolute numbers of mRNA target molecules per unit of starting RNA without using internal controls.

If the two conditions are in effect (i.e., E is constant and reactions are not reaching the plateau phase), **Eq. 3.1** indicates that there is a linear relationship between the logarithm of the starting amount of target mRNA (or cDNA) (included in A_0) and the logarithm of the amount of amplification product generated (A). This relationship is illustrated in the graph of **Fig. 3**. A linear relationship between log A and log A_0 has also been shown to exist empirically for values of A ranging over 2–3 orders of magnitude (14–16). In one case, this relationship was even found to hold for values of A differing by four orders of magnitude *(11)*.

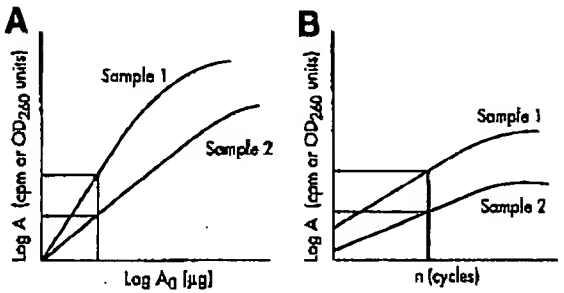

Fig. 4. Two methods for determining relative differences in the initial amount of target in two samples. **(A)** Titration method and **(B)** kinetic method.

3.1. Determining Relative Differences In N_0 Between Two or More Samples

Two forms of experimental analysis, titration and kinetics, can be used to estimate the relative initial amounts of target mRNA or cDNA in two samples—when the amplification efficiencies are the same for the two samples and the data are collected before the reactions begin to reach the plateau phase.

3.1.1. Titration Analysis

A titration analysis is performed by making a dilution series, or titration, of RNA or cDNA, amplifying by PCR, and quantifying the signals produced (defined as A). **Fig. 4A** shows idealized data collected from this type of experiment, graphed as log A as a function of log A_0. Because of the linear relationship between log A_0 and log A, and because the amount of target mRNA or cDNA is a constant proportion of the total starting material (A_0) for each of the various dilutions of a given sample, the relative difference in N_0 is proportional to the difference betwen the slopes of the two curves. Thus, a value of log A_0 is chosen on the X (horizonal) axis of the graph and the corresponding values of log A are then extrapolated for both curves, as shown in **Fig. 4A**. The difference between the two values of log A determined in this manner from the graph is equivalent to the relative difference in N_0 for the two samples. Singer-Sam et al. *(16)* used this method to determine the relative changes in mRNA levels for several phosphoglycerate kinases and phosphoribosyl-transferases during mouse spermatogenesis.

3.1.2. Kinetic Analysis

A more commonly used alternative to titration analysis is comparative kinetic analysis. To perform a kinetic analysis, values of A are determined for a number of consecutive amplification cycles (n) for two samples. **Fig. 4B** shows idealized data from an experiment of this type, plotted as log A vs n. The curves are consistent with **Eq. 3**.

To determine the relative difference in N_0 between the two samples, a value of n is chosen at a point where the two curves are parallel (suggesting equal values for E), and the value of log A is extrapolated from this value of n for each curve. At this point, the difference between the two values for log A is directly proportional to the difference of log A_0 between the two samples. Moreover, the difference of log A_0 between the two samples is equal to the difference of log N_0 between the two samples. Hence, this method can be used to determine the difference in the initial number of target molecules, but not the actual number of starting target molecules.

Comparative kinetic analyses have been used to accurately detect 2- to 10-fold changes in mRNA levels. For example, Solomon et al. *(17)* used this approach to examine differences in the levels of apolipoprotein mRNA in normal and atherosclerotic blood vessels. Dallman et al. *(18)* used a similar strategy to examine the influence of tissue transplantation on cytokine mRNA levels.

3.2. Using Linear Regression Analysis to Determine the Absolute Value of N_0

Equation 3 describes a linear relationship in the format, $y = mx + b$, whose slope (m) has the value of log (1 + E) and whose y-intercept (b) is N_0. This allows estimation of the value of N_0 graphically. When the value of E is known, the value of N_0 can be determined from a linear regression analysis of the plotted data. Experimentally, a kinetic study is performed in which a constant amount of starting cDNA is amplified by PCR. During consecutive cycles, the number of product molecules, N, is determined. In this method it is necessary to calculate N, and not simply A. With the data graphed as log N vs n, E can be calculated from the logarithm of the slope, and N_0 can be derived from the y-intercept (**Fig. 5**).

This method was recently used by Wiesner *(7)* to estimate the number of α- and β-myosin heavy-chain mRNA molecules per unit of total RNA extracted from rat ventricle tissue. The authors also were able to calculate the number of mRNA molecules per cell, taking into account the yield of RNA and the number of myocytes per gram of tissue.

4. Quantitative PCR Using Internal Standards

Thus far, a variety of methods for using quantitative PCR to determine relative initial levels of target mRNAs, and one method for estimating the absolute

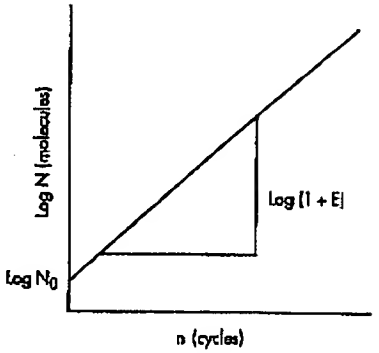

Fig. 5. Determination of initial amount of target (N_0) and efficiency of amplification (E) using linear regression. Note that the slope = log (1 + E) and the y-intercept = E_0.

starting number of target molecules have been discussed. However, in all of these methods, variations in amplification efficiency (E) may complicate the interpretation of results. To correct for tube-to-tube variations in amplification efficiency, most investigators use internal amplification standards. Two types of internal standards can be used: an endogenous sequence or gene transcript that is normally present in the sample, or an exogenous fragment added to the amplification reaction.

4.1. Amplification of an Endogenous Sequence as an Internal Standard

An endogenous sequence, known to be present at constant levels throughout a series of samples to be compared, can be used as an internal standard in quantitative PCR reactions. Endogenous mRNA standards, typically for housekeeping genes or genes that are structurally or functionally related to the target mRNA *(19)*, have been used to determine relative levels of specific mRNAs *(13,20–22)*. Furthermore, endogenous single-copy gene sequences have been used as internal standards to determine relative gene copy numbers *(5,23,24)*. Finally, there is at least one case where ribosomal RNA was used as an endogenous internal standard for quantitation of mRNAs *(25)*.

In this approach, the endogenous standard sequence is amplified using a second pair of gene-specific primers, either in two separate PCR reactions, or in the same reaction as the target sequence. The ratios of the amount of PCR products generated by target and endogenous standard sequences in the different samples are then determined and compared. As with the methods described previously, the data from this type of experiment must be obtained before the amplification reactions reach the plateau phase. The data can be collected either

from a titration of the sequences to be amplified, or by kinetic analysis, to ensure that signals are derived only from the exponential phase of the amplification.

The relative initial amounts of a target sequence and the endogenous standard (i.e., the ratio N_0t/N_0s) can be determined from **Eq. 4** (derived from **Eq. 2**). (The subscripts "*t*" and "*s*" refer to the target and standard sequences, respectively.) Values for the efficiency of amplification (E) for the target and standard may be calculated from the slope of a graph of log N as a function of cycle number (n) (*see* **Subheading 3.2.**). Note that when the amplification efficiencies of the two reactions—target and standard—are identical, i.e., $Et = Es$, the analysis is greatly simplified *(3)*.

$$N_0t / N_0s = Nt(1 + Es)n / Ns(1 + Et)n \quad (4)$$

where N_0t = The initial number of target molecules, N_0s is the initial number of standard molecules, Nt is the number of amplified target molecules, Ns is the number of amplified standard molecules, Et is amplification efficiency of the target, Es is amplification efficiency of the standard, and n is the number of amplification cycles.

Even without a full mathematical analysis—and even in cases where Es does not equal Et—it has been shown empirically that endogenous mRNAs can be used to normalize target mRNA levels between samples to be compared. Thus, instead of determining the ratio of the intial absolute amounts of target and standard using linear regression, the relative amounts of PCR products generated by the target and standard templates in different samples is simply compared. Although it has not been shown theoretically, Horikoshi et al. *(13)* suggested that if the internal standard mRNA is expressed at the same level in two samples, the ratio of PCR products generated from the target and standard should indicate the relative level of expression of the target mRNA in those samples. Furthermore, it may be true that if the target and standard are amplified in the same tube, tube-to-tube variations in amplification efficiency (for example, caused by pipeting error, sample impurities, variation in the heating block, or partially degraded RNA) may be minimized as well.

This type of approach has been experimentally validated by performing PCR on mixtures of DNA. For example, Horikoshi et al. *(13)* mixed specific ratios of DNA preparations from two cell lines, one with a documented 18-fold amplification of the dihydrofolate reductase (DHFR) gene and the other carrying the gene as a single copy. In this case, two independent PCR amplifications were performed on each sample using DHFR and β-actin primers, respectively, in separate reactions; the β-actin sequence served as a single-copy standard. Amplified products were obtained under conditions in which the amount of product was still increasing linearly with increasing amounts of starting sample (A_0). The ratio of DHFR to β-actin PCR products obtained from the mixtures differed by only ~30% from the predicted theoretical values.

In the aforementioned experiment, the amplification of standard and target sequences was conducted in separate PCR reactions. However, a close correlation between predicted and observed target levels was similarly found by Neubauer et al. *(22)*, who performed both amplifications in a single PCR reaction in a method they referred to as differential PCR. In this case, the authors were investigating the loss of the β-interferon gene in chronic myelogeneous leukemia; the target was the β-interferon gene and the standard was the γ-interferon gene. They were able to detect changes as small as 2:1 and 3:2 in the ratio of the two genes using this method. Co-amplification also was used by Chamberlain et al. *(23)* to examine exon deletions in the Duchenne muscular dystrophy locus. In an approach they called multiplex DNA amplification, they simultaneously amplified (in one tube) six exons, each with a different set of primers. In another example, Kellogg et al. *(5)* corrected for the effects of variable amplification efficiency of an HIV-1 DNA template in several samples by using a single-copy gene from the HLA locus as a reference standard.

Many examples of the use of endogenous mRNA standards to determine relative levels of specific mRNAs (in the same tissue) can be found in the literature. The first group to use this approach was Chelly et al. *(3)*, in a study of dystrophin gene expression in different muscle tissues. Chelly et al. used aldolase A mRNA as the internal standard, and they performed the mathematical analysis, including calculation of amplification efficiencies, described at the beginning of this section. Noonan et al. *(19)* studied the relative expression of the multiple drug resistance gene (*mdr*-1) in tumor cells by normalizing PCR data to β_2-microglobulin mRNA. Horikoshi et al. *(13)* investigated expression of thymidylate synthase mRNA in tumor samples using both β_2-microglobulin and β-actin mRNA as endogenous standards. Murphy et al. *(20)* utilized both target titration and kinetic strategies to examine *mdr*-1 mRNA levels in tumor cells. Finally, Kinoshita et al. *(21)* examined levels of T-cell leukemia virus type I by performing a detailed kinetic PCR analysis that used β-actin mRNA as the endogenous standard.

Perhaps the greatest advantage of using the expression of an endogenous sequence as an internal standard is that the reference mRNA and the target mRNA are usually processed together for the entire duration of the experiment—from RNA extraction through PCR amplification. This minimizes differences in RNA yield between samples—an important advantage, particularly for analysis of small tissue samples where the quantities of RNA are too small to measure by UV spectrophotometry. In addition, if the entire population of mRNA is converted to cDNA by the use of oligo(dT) primers or random hexamers, the overall efficiency of cDNA synthesis also is somewhat normalized.

Notwithstanding the advantages to this approach, several complications may arise when amplification of endogenous mRNAs is used for semi-quanti-

tative analysis. For this method to be reliable, the level of expression of the reference standard must be the same in each sample to be compared and must not change as a result of the experimental treatment. Unfortunately, few if any genes are expressed in a strictly constitutive manner. This is even the case for many housekeeping genes, including β-actin *(26,27)*. Therefore, the level of the mRNA used as the endogenous standard must be examined very carefully to ensure its constancy among all of the experimental conditions studied.

Another challenge of this approach is to obtain values of *At* and *As* before the amplification reactions reach the plateau phase, especially when the relative levels of expression of the standard and target sequences differ greatly. For example, if β-actin mRNA is used as the internal standard, it may be present at a much higher level than the target transcript, and amplification of the control may approach plateau phase well in advance of the target sequence. Indeed, Murphy et al. *(20)* found that their internal standard mRNA, β_2-microglobulin, entered the plateau phase before the target, *mdr*-1 mRNA, was even detectable. One solution to this problem involves simply waiting until later stages of the amplification before adding the primers for the endogenous standard *(21)*. Other researchers used gene-specific primers to synthesize cDNA from the control and target mRNAs in separate tubes and then mixed dilutions of the control and target cDNAs before performing multiplex PCR *(25)*.

Interference is a frequently observed problem when more than one set of primers is used in the same PCR reaction. For example, when Murphy et al. *(20)* added both β_2-microglobulin and *mdr*-1 primers to the same PCR reaction, they observed a premature attenuation of the exponential phase of both PCR amplifications. At Clontech (Palo Alto, CA), researchers have observed similar results; the amount of product generated (from either the target, the standard, or both) is often dramatically reduced when both sequences are amplified in a single reaction. In fact, primer pairs that function truly independently seem to be the exception rather than the rule.

4.2. Amplification of an Exogenous Sequence as an Internal Standard

Exogenous sequences can also be used as internal PCR standards. In this approach, an exogenous mRNA or DNA standard is added to the target sample and amplified simultaneously with the target transcript in a single PCR reaction mixture. The exogenous standard can be either a synthetic RNA added to the reverse transcription reaction or a DNA, not normally in the target sample, that is added directly to the PCR reaction.

The theory behind use of added exogenous gene sequences as internal standards is similar to that described earlier for endogenous reference sequences. With both types of internal controls, the amount of amplified standard can be

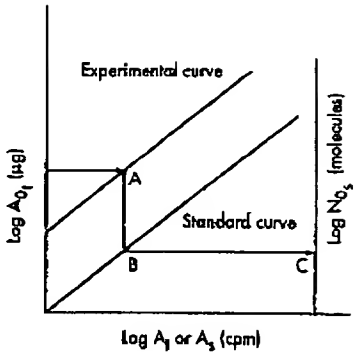

Fig. 6. Use of a standard curve, derived from an exogenously added internal standard, to quantitate initial amount of target.

quantified after the experiment, and the change in the amount of standard is proportional to the change in the amount of target. However, there is a significant advantage in using an exogenously added sequence as the internal control; namely, the initial amount of standard used in the PCR reaction is precisely known. This makes it possible to calculate the absolute level of target mRNA or cDNA present in the original sample.

A common method of obtaining quantitative results from PCR with an exogenous standard involves generating a standard curve from the data collected. This method was first described by Wang et al. *(28)*, who quantified changes in the levels of several cytokine mRNAs in stimulated macrophage cells using a synthetic internal RNA standard. In this approach, the RNA standard shares primer binding sites with the target RNA, but posseses a different "stuffer" sequence and an oligo(dT) tail. A known amount of the RNA standard is mixed with a known quantity of RNA sample (measured in micrograms, for instance) and reverse transcribed. A series of PCR reactions is then set up with dilutions of the cDNA. Because the titration is performed on a defined mixture of the target and standard mRNAs, and because the mixtures are not titrated against one another, this is not a competitive reaction (competitive PCR is discussed later). This strategy simply allows the generation of two titration curves: one for the standard RNA and one for the target RNA.

Following PCR, the amounts of PCR products obtained from the standard (*As*) and target (*At*) sequences are determined, and two curves are plotted, as illustrated in Fig. 6. The RNA standard curve is generated by plotting the logarithm of the starting number of RNA standard molecules (log N_0s) on the right vertical axis as a function of the logarithm of the amount of amplified standard product (log *As*). The target RNA curve is generated by plotting the logarithm

of the initial amount of RNA sample (log A_0t) on the left vertical axis as a function of the logarithm of the amount of target amplification products (log At). To determine the number of target mRNA molecules per unit of total RNA, a value of A_0t is chosen in the region where the curves are parallel (e.g., where values for E are identical). A line is drawn from that point (labeled A in **Fig. 6**) down to the internal standard curve (point B), and from point B, a line is drawn across to the right vertical axis (point C). The value at this point is taken as the starting number of target molecules, N_0t, in each microgram of total RNA. If the amount of total RNA per cell is known, the actual number of target mRNAs per cell can also be calculated. In the study by Wang et al. *(28)*, changes in mRNA levels of threefold or less were reproducibly discernible. Also, the results correlated well with data obtained from a Northern blot analysis. A similarly close correlation between this method of quantitative PCR and Northern blot analysis was found by Prendergast et al. *(29)*.

A critically important requirement of this type of experiment is that the value of E be the same for both the target and standard mRNAs. This can be accomplished by designing the standard to contain the same primer binding sequences as the corresponding target mRNA. In many cases this is sufficient to make Es equal to Et. Additional requirements for using exogenous standards are that the PCR products be of similar size and under 1 kb. The author has observed, as did Wang et al. *(28)*, that the primer sequences have the greatest effect on amplification efficiency when the sizes of the amplified sequences are similar. Wang et al. showed that the amplification efficiency of an RNA standard was the same as that of its corresponding target even though the sequence between the shared primer binding sites was completely different. Of course it is important that no regions of significant secondary structure differ between the target and standard RNA sequences. Differences in efficiency still may exist, so this parameter should always be examined before drawing firm conclusions from each study.

To calculate the absolute initial number of target molecules (A_0t), the initial number of standard molecules (A_0s) must be known, and a method to differentiate between the number of amplified standard and target molecules (As and At, respectively) must be available. The most common technique used to distinguish between As and At is to make their sizes sufficiently different such that they can be resolved by polyacrylamide or agarose gel electrophoresis. Probe hybridization also can be used if the sequence between the two primer binding sites differs. In some cases, different restriction sites within the sequences between the primer binding sites can be used to differentiate target from standard simply by digestion with an appropriate restriction endonuclease prior to gel electrophoresis.

Since the study by Wang et al. was published, several reports have described the construction of exogenous RNA and DNA internal standards that differ from target sequences only by the presence or absence of small introns or restriction sites *(30–32)*. In these cases, there is little doubt that the amplification efficiencies of the standard and target sequences will be the same.

5. Competitive PCR

Competitive PCR also uses an exogenous template as an internal standard. However, the amplification takes place in a truly competitive fashion because the standard and target sequences actually compete for the same primers. In competitive PCR, a dilution series is made of either the target sequence or the standard sequence, and a constant amount of the other component is added to each of the reactions. Quantification is performed after competitive amplification of the entire series of reactions and is achieved by distinguishing the two PCR products from each tube by differences in size, hybridization properties, or restriction enzyme sites. An important advantage of competitive PCR is that, because the ratio of target to standard remains constant during the amplification, it is not necessary to obtain data before the reaction reaches the plateau phase.

In competitive PCR, the competitor fragment (usually DNA) takes the place of the standard described in the experiments discussed in the previous sections. It will still be called the standard, and the symbol "s" will be used to designate it in equations. When the amplification efficiencies of the target and standard molecules are the same, **Eq. 4** can be simplified to **Eq. 5**.

$$N_0t / N_0s = Nt / Ns = At / As \quad (5)$$

where N_0t is the initial number of target molecules, N_0s is the initial number of standard molecules, Nt is the number of amplified target molecules, Ns is the number of amplified standard molecules, At is the amount of amplified target (in cpm or OD_{260} U), and As is the amount of amplified standard (in cpm or OD_{260} U).

Thus, for any value of n, the initial ratio of target to standard is equal to the ratio of their amplification products (i.e., Nt/Ns or At/As). This has been demonstrated both theoretically *(33)* and empirically *(34)*. Therefore, if the standard and target sequences amplify with the same efficiency, the absolute initial amount of target cDNA (and in turn target mRNA), can be determined by allowing known amounts of standard (DNA) molecules to compete with the target for primer binding during amplification.

In the competitive PCR method illustrated in **Fig. 7**, a dilution series of the DNA standard (referred to in the figure as the "MIMIC"*) is made, and these dilutions are added to a series of PCR reactions containing a constant amount of sample cDNA. Following PCR, the amplification products are analyzed by

*The use of PCR MIMICs is discussed in **Subheading 5.2.1.**

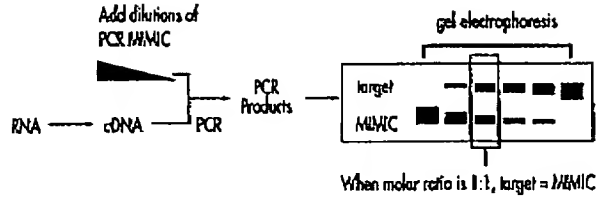

Fig. 7. Schematic diagram of competitive PCR utilizing a competitor DNA fragment (PCR MIMIC) differing in size from the target sequence. A dilution series of the competitor is added to a constant amount of cDNA. Following amplification, samples of the PCR products are resolved by gel electrophoresis, and the yields of amplified competitor and target products are quantified. The relative amounts of target product and MIMIC product in each sample are compared. The initial amounts of target and competitor are assumed to be equal in those reactions where the molar ratio of target and competitor products are judged to be equal (after correction for size differences). Because the amount of competitor added to each PCR reaction is known, the absolute initial amount of target can be determined. If the competitor is a synthetic RNA, a dilution series of the competitor is added to a constant amount of sample RNA before the reverse transcription step.

gel electrophoresis, and the amount of products generated by the standard (*As*) and the target (*At*) are determined for each individual reaction. The logarithm of the ratio of *At*/*As* is graphed as a function of the logarithm of the initial molar amount of the standard (N_0s) (Fig. 8). The initial amount of target cDNA (N_0t) is extrapolated from the graph, assuming that N_0t is equal to the amount of the standard (N_0s) added when an equimolar ratio of the two types of products is generated (i.e., where the log of *At*/*As* = log of 1/1 = 0). Note that if there is a difference in the size of the standard and the target sequence, N_0t does not precisely equal N_0s (because longer sequences incorporate more label than shorter ones). Thus, a corresponding correction must be made in the calculation of N_0t.

In general, when determining absolute initial amounts of mRNAs by competitive PCR using standard DNA fragments, one must take into account the fact that the efficiency of reverse transcription is <100%. The efficiency of cDNA synthesis using oligo(dT) as a primer for cDNA synthesis has been reported to be 40–50% *(34,35)*. Thus, calculations such as those described previously underestimate the number of mRNA molecules present in a given sample.

5.1. Homologous Competitor Fragments

Becker-André and Hahlbrock *(31)* and Gilliand et al. *(32)* were the first to describe competitive PCR using homologous competitor fragments. Gilliand et al. used two types of internal standard: a genomic fragment corresponding to

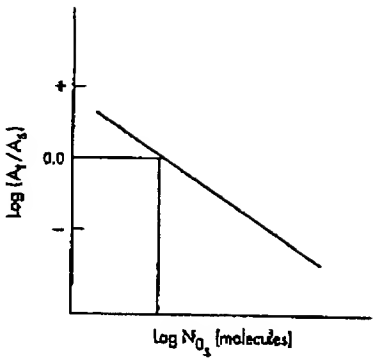

Fig. 8. Analysis of the results of a competitive PCR experiment, such as that illustrated in Fig. 7. The log of the ratio of amplified target to competitor products is graphed as a function of the log of the known amount of competitor added to the PCR reaction. Note that when the molar ratio of target and competitor is equal to 1, the log of that ratio equals 0.

the target mRNA sequence, but containing a small intron (thus yielding a PCR product slightly larger than the target mRNA); and a cDNA which was modified to contain a unique restriction site. In the latter case, PCR products were digested with the appropriate restriction enzyme before electrophoresis to differentiate between target and competitor products. To generate their internal standard, Becker-André and Hahlbrock used an in vitro transcribed mRNA designed to be identical to the target mRNA sequence except for the addition of a unique restriction site. They added different amounts of the competitor RNA into reverse transcription reactions containing a constant amount of target RNA.

Although it may be relatively easy to perform restriction endonuclease digestions to differentiate between target and competitor products, construction of such competitor fragments is often not a trivial matter. Unless there is a known small intron in the target gene, the construction of homologous competitors can require time-consuming site-directed mutagenesis and multiple cloning steps. However, recently several clever methods that use simple PCR amplification with composite primers *(36–38)* have been developed to generate homologous DNA standards. These methods can be extended to yield homologous competitor RNAs as well.

One potential problem with the use of competitor fragments that are homologous to the target is that during later stages of PCR, when the concentration of products is high, heteroduplexes can form between the standard and target sequences. This can complicate quantification of the PCR products derived specifically from the target or the standard, particularly when restriction

enzyme digestion is required to distinguish between them. Therefore, heterologous DNA standards may be preferable (*see* **Subheading 5.2.**).

Although heteroduplex formation often interferes with obtaining accurate quantitative results from competitive PCR, in a novel approach described by Henco and Heibey *(39)* it is the heteroduplexes that are actually quantified. A known quantity of an internal standard, which is identical to the target except for a single nucleotide, is added to a dilution series of the target sample. Following PCR, a trace amount of radioactively labeled standard is added to the PCR products. The mixture is denatured and allowed to re-anneal; the labeled standard anneals to both target and standard sequences as a tracer. The homoduplexes and heteroduplexes are then resolved by temperature-gradient gel electrophoresis, and the amount of material in the heteroduplex (reflecting the amount of amplified target) is quantified.

5.2. Heterologous Competitor Fragments

DNA fragments that share the same primer template sequence but contain a completely different intervening sequence can also be used for competitive PCR. Überla et al. *(40)* prepared fragments for competitive analysis by amplifying genomic DNA fragments from another species with a low annealing stringency. Siebert and Larrick *(41)* ligated the primer template sequences to a nonhomologous DNA fragment to generate DNA standards (competitor fragments). More simply, the competitive DNA standard can be obtained by amplification of a heterologous DNA fragment using composite primers.

5.2.1. Generation of PCR MIMICs

One type of heterologous competitor fragment, PCR MIMIC (Clontech), is available commercially. PCR MIMICs are generated by two successive PCR amplifications as shown in **Fig. 9.** In the first PCR reaction, a heterologous DNA fragment is amplified using two composite primers. One composite primer contains the upstream primer for the target sequence linked to a 20-mer that anneals to one strand of the heterologous DNA fragment. The other composite primer contains the downstream primer for the target sequence linked to a 20-mer that anneals to the opposite strand of the heterologous DNA fragment. The two composite primers are used to amplify a small fragment of the heterologous DNA. During amplification, the target-specific primer sequences are incorporated into the PCR product. This PCR product is diluted and used to perform a second PCR amplification with primers for the target gene only. In this way the entire target primer sequences are incorporated onto the ends of the heterologous DNA fragment.

The PCR product, the newly generated PCR MIMIC, is purified by passage through a spin column that removes PCR reaction components and primers. The quantity of PCR MIMIC obtained is then determined either by measuring

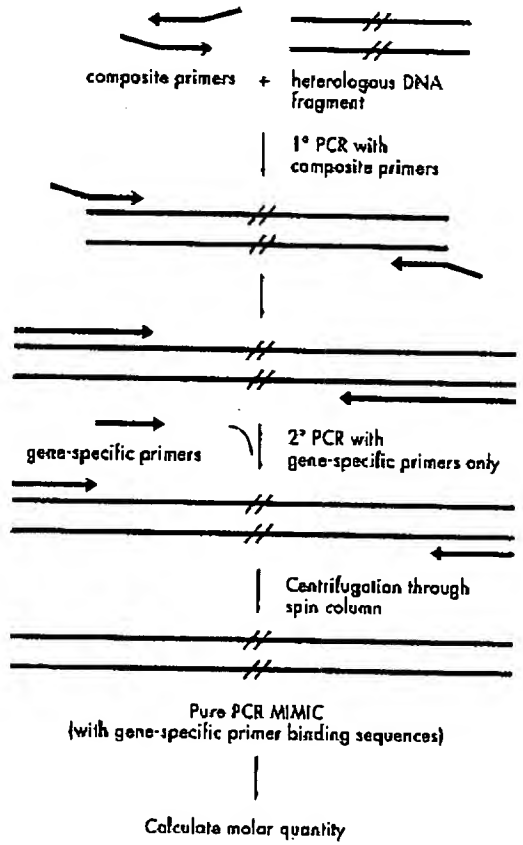

Fig. 9. Flow chart illustrating the generation of competitive PCR MIMICs. Note that the composite primers are composed of two sections; the 3' portion anneals to the heterologous DNA fragment and the 5' portion anneals to the specific target gene.

the absorbance at 260 nm or by running an aliquot of the PCR MIMIC on a gel and comparing the intensity of the band to a dilution series of DNA markers containing known quantities of DNA. The mass quantity is then converted to molar quantity using the approximation that 1 ng of a 300-bp DNA fragment is equal to 5×10^3 attomol (1 attomol = 10^{-18} mol).

A typical yield of PCR MIMIC, approx 200 ng, is enough to perform hundreds of competitive PCR experiments. Therefore, in practice a single determination of MIMIC yield can be used for many experiments. In this way inaccuracies in yield determination will not affect the determination of relative changes in mRNA levels (from a series of samples) by competitive PCR.

PCR MIMICs are designed so that the size of the PCR product generated from them is either slightly larger or slightly smaller than the PCR product generated from the target sequence. MIMICs of different sizes can be made simply by designing the composite primers so they anneal to different regions on the heterologous DNA fragment.

5.2.2. Use of RNA MIMICs

The method for generating competitive PCR MIMICs can be extended to heterologous RNA MIMICs to explicitly control for the cDNA synthesis step. To generate an RNA MIMIC, an RNA polymerase promoter and poly $(A)^+$ tail can be incorporated into the PCR product using composite primers designed for that purpose. In vitro transcription of the PCR product generates synthetic RNAs that contain the target primer sequences and a poly $(A)^+$ tail. RNA samples can then be titrated with the RNA MIMIC during reverse transcription. Transcriptional promoters have been successfully incorporated into PCR products via primer sequences *(13)*, and recently, competitive RNA fragments have been generated by this method *(42)*.

6. Summary of Quantitative PCR Methods

PCR is an exponential reaction in which small variations in amplification efficiency can yield large changes in the amount of products. In addition, later cycles of PCR exhibit the plateau effect, in which the rate of amplification slows and eventually levels off. These characteristics of PCR can make it difficult to obtain quantitative data. However, if specific conditions and proper controls are used, quantitative information about mRNA levels can be obtained. Of the various quantitative RT-PCR techniques currently in use, competitive PCR is often the method of choice. Competitive PCR is accurate enough to discern differences in mRNA levels of two- to threefold or smaller. This is comparable to the accuracy of quantitative methods that use either endogenous or exogenous internal standards in noncompetitive experiments.

Some investigators have observed that careful kinetic analyses can be used to determine initial concentrations of mRNAs by linear regression analysis without internal controls. At least one group, Singer-Sam et al. *(16)*, obtained satisfactory results without using either internal controls or kinetic analysis. Nonetheless, many have found it necessary to include internal controls to address the problem of tube-to-tube variation in amplification efficiency. Internal controls can be endogenous mRNA or exogenous mRNA added to the cDNA synthesis reaction. In addition, exogenous standards can be designed with the same or different primer annealing sequences as their target molecules. Each type of internal control has advantages and limitations.

One clear advantage of using endogenous internal mRNA controls is that the yield of RNA and the efficiency of the reverse transcription can vary to some extent without loss of accuracy. However, preliminary studies must be performed to ensure that the endogenous control mRNA does not change during the experiment. This must be tested because many genes, including many housekeeping genes whose expression may seem unrelated to the experimental conditions, may nevertheless vary in the experimental conditions being compared. In addition, the data must be collected before the amplification reaction reaches the plateau phase. This can be difficult if the endogenous control gene is expressed at a different level than the target gene or if their relative amplification efficiencies differ greatly.

Exogenous internal standards that share the same primer annealing sequences with the target allow calculation of the absolute amount of target mRNA, as determined by Wang et al. *(28)*. A similar method, termed competitive PCR, circumvents many of the disadvantages of the other quantitative methods. Competitive PCR can be used to measure relative changes in mRNA levels as well, for example, in gene regulation studies. However, two conditions must be met to use competitive PCR. One, the molar quantity of the competitor RNA or DNA must be known. (Usually this is not a problem because it can be measured by UV spectrophotometry.) Two, the amplification efficiency of the competitor and target must be identical. This is often true because the standard and target possess the same primer binding sequences. If the standard is a DNA fragment, the efficiency of the reverse transcription also must be considered.

Perhaps the most important advantage of competitive PCR is that useful data can be obtained during the entire course of amplification—even after the reaction has reached the plateau phase. This is not the case for methods using internal standards without competition between the standard and target molecules. Recently, however, Pannetier et al. *(43)* cautioned that competitive PCR may not provide accurate results when the sequences of the target and standard molecules are completely different (except for the primer sequences) and when the data are collected well after the plateau phase of the reaction. As stated previously, an examination of amplification efficiency is warranted.

Advantages and limitations of using homologous and heterologous competitor DNA fragments as internal standards for quantitative PCR have been discussed. In summary, homologous competitor fragments have the same amplification efficiency as their corresponding target but can form heteroduplexes that can complicate the measurement of PCR products. Heterologous competitor fragments, on the other hand, cannot form heteroduplexes, but their amplification efficiencies must be shown to be equal (or very similar to) that of the target.

7. Conclusions

It is possible to obtain quantitative information about specific mRNA levels using RT-PCR. The ability to accurately measure of gene expression in small amounts of tissue or in mixed cell populations will considerably expand future

applications of PCR, both in research laboratories as well as in clinical settings. For example, quantitative PCR will be used increasingly in gene expression studies aimed at understanding the basic mechanisms controlling differentiation, development, immunity, and tumorigenesis. In a clinical application, competitive PCR has already been used to quantitate HIV transcripts in patient samples *(44)*. In the future, quantitative RT-PCR can be expected to aid the diagnosis and monitoring of many human diseases.

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